Prevalence of Resistance-Associated Mutations in Human Immunodeficiency Virus Type 1-Positive Individuals Failing HAART in Rio de Janeiro, Brazil

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We investigated the occurrence of HIV-1 antiretroviral resistance in individuals failing to respond to highly active antiretroviral therapy (HAART) attended by RENAGENO from 2001-2004. One hundred and seventeen patients were selected for this study; their plasma viral RNA was extracted and the PR and RT genes sequenced to examine subtype, genetic polymorphisms and mutations associated with resistance to antiretroviral drugs. HIV-1 sequence analysis showed that 86/100 (86%) were infected with subtype B, 7/100 (7%) with subtype F and 7/100 (7%) with RT/PR hybrid forms (2 D/B, 2 F/B, 2 B/F and 1 D/F). In 14 (12%) of the samples, the subtype was not determined. The prevalence of resistance mutations was high (93.1%), mainly in the RT gene. The most prevalent resistance mutations were: M184V (60.7%), T215Y (49.6%) and M41L (46.7%) in the RT gene and L90M (19.6%), M46I (16.2%) and D30N (12.8%) in the PR gene. The frequency of resistance mutations tended to increase from the first to the second therapeutic scheme failure (p=0.079); but it stabilized after subsequent failures (p=0.875). Our finding of a high frequency of drug resistant HIV-1 samples supports the need for continuous genotypic monitoring of patients failing HAART.

Key words: HIV-1, HAART, reverse transcriptase, protease, resistance.

Antiretroviral therapy (ARV) developed to control human immunodeficiency virus (HIV) infections includes nucleosidic and non-nucleosidic reverse transcriptase inhibitors (NRTIs and NNRTIs, respectively) and protease inhibitors (PIs) [1,2]. In order to improve clinical and virological responses, current therapy is composed of a triple-drug combination of at least two of these drug classes; this is known as highly active antiretroviral therapy (HAART) [3]. In Brazil, more than 130,000 people have free access to ARV [4]. This state policy has helped reduce AIDS incidence by 50% during recent years and has improved the quality of life of HIV-positive individuals in our country [5]. However, HIV resistance to ARV, leading to HAART failure, is inevitable in the course of infection [5], as is the transmission of resistant strains [6]. Resistance is explained by intense viral replication in the host (10^{10} particles per day) [7], associated with random mutations occurring in the viral genome, due to reverse transcriptase (RT)-polymerase errors (10^{-4} per incorporated nucleotide) [8,9]. The result of this combination is the emergence of different but related HIV genetic populations, known as quasispecies [10].

When mutations occur in certain sites of the protease (PR) and RT genes of the *pol* region of the HIV genome [11], which are responsible for vital enzymatic functions, resistance to ARV drugs is possible [12]. The consequence of HIV-resistance is therapeutic failure, resulting in a plasmatic viral load boost and the appearance of opportunistic diseases [13,14]. Weak adhesion to treatment and late detection of viral resistance also reduce the odds of therapeutic regimen success and increases the transmission of resistant strains [15].

In order to evaluate the extension of resistance and distribution of subtypes in our country, the Brazilian government created the National Network for HIV Genotyping (RENAGENO) in 2001 [16], involving reference laboratories and a team of specialists in HIV resistance. Genotype testing has been found to be beneficial for guiding therapy in patients with HIV infection and virological failure [17-19]. We investigated the occurrence of HIV-1 resistance by analyzing RT and PR genes in individuals failing HAART.

Material and Methods

Patients

One hundred and seventeen patients selected for genotyping tests from 2001 to 2004 were attended by one of the RENAGENO-associated units, the Viral Load Laboratory of Federal University of Rio de Janeiro. Informed consent was obtained from each individual after the nature and possible consequences of the study had been fully explained. Selective criteria for genotyping tests were based on HIV therapeutic failure, based on parameters established by the Brazilian Ministry of Health and viral load counts above 5,000 copies/mL. A brief profile of patients failing HAART was also developed.

RNA Isolation, Amplification and Genotyping

Plasma viral RNA isolated and purified by ultra centrifugation (120,000g for 120 min.) was submitted to a first round reverse transcription with MuLV enzyme, followed by a hot start PCR with the AmpliTaq® Gold. The DNA product that was generated corresponds to the *pol* fragment covering the PR and RT genes. Viral load was quantified [Nuclisens HIV-1 QT (NASBA Diagnostics, Biomerieux, Boxtel, NL)] before genotyping the samples with seven

Received on 19 February 2008; revised 18 September 2008.
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The Brazilian Journal of Infectious Diseases 2008;12(5):380-384.

internal primers using the BigDyeÔ Terminator chemistry (Applied Biosystems, US). The sequencing products were visualized with an ABI 3100 Genetic Analyzer (Applied Biosystems, US).

Sequence Alignment and Phylogenetic Analysis

The generated RT and PR sequences were tested against the reference set from the Los Alamos database (hiv-web.lanl.gov) for subtyping analysis. Sequences were aligned using the ClustalW algorithm and then manually corrected. Phylogenetic trees used in later analyses were built using the maximum likelihood (ML) method in PAUP*4b10.

Identification of Resistance Mutations

Sequences were submitted to the resistance test program available at Stanford University (http://hivdb.stanford.edu). This program analyses differences in amino acid sequences, including positions that contain a mixture of wild type and mutant residues, according to the consensus statement of the International AIDS Society-USA Resistance Panel [20].

Statistical Analysis

Databank and statistical analyses were done with Epi-info 2000 software. Descriptive statistics included frequency distribution, means and correlations. We used a chi-square test to examine associations of categorical variables. P values <0.05 were considered significant.

Results

One hundred and seventeen patients failing HAART attended by RENAGENO participated in this study. The general profile of this group is summarized in Table 1. The male gender predominated (63.2%); however, no significant gender differences were found in the number of therapeutic regimens (p=0.680), distribution of viral subtypes (p=0.120) or presence of resistance mutations. The period from 1996 to 1999, especially 1997, corresponded to a major ARV therapy initiation effort (37.6%); as expected, the period under ARV treatment was positively related with the number of therapeutic regimens used (R^2 =0.868). HIV-1 subtyping of patients failing HAART revealed that subtype B was the most common; it was found in 86/117 (73.5%) samples, followed by F in 7/117 (6%) and PR/RT hybrid forms (D/B; F/B; D/F; B/F) in 7/117 (6%) samples. In 12% of the samples, the subtype could not be determined. Subtype C was not detected in this group.

In 109/117 (93.1%) samples, we identified mutations to one or more drug classes (NRTIs, NNRTIs and PIs); a large number of resistance mutations occurred in the RT gene 109/117 (93.1%), in comparison with 48/117 (41.0%) in the PR gene (p<0.001). We found that 6.8% of the samples had no resistance mutations. The most frequent ARV-associated resistance mutations were: M184V (60.7%), T215Y (49.6%), M41L (46.7%), L210W (30.8%) and K103N (27.3%) in the RT gene and L90M (19.6%), M46I (16.2%), D30N/N88D (12.8%) and I54V (10.2%) in the PR gene. Table 2 shows the effects of these mutations on ARVs. Secondary mutations also predominated in PR and RT genes and could be found in all of the samples 117/117 (100%). The most frequent were: L63P (47.0%), L10I/F (34.2%), V77I (29.0%), A71V/T (26.5%) and M36I (21.3%) in the PR gene and I293V (62.3%), K122E (55.7%), I135T (55.7%), A272P (54.1%) and R211K (42.6%) in the RT gene.

The number of resistance mutations tended to increase from the first (mean of 3.18 resistance mutations/sample) to the second therapeutic failure (4.39 resistance mutations/sample) among individuals failing HAART (p=0.079); although the difference was not significant. The number of resistance mutations per sample apparently decreased or stabilized following therapeutic failures (p=0.875, Table 3).

Discussion

In Brazil, ARVs have been shown to be effective [4]. However, these drugs also increase the possibility of developing resistance in patients with incomplete viral suppression [21,22]. In 2001, the Brazilian Ministry of Health created the National Network for HIV Genotyping (RENAGENO) to monitor HIV resistance and to examine subtype distribution over the country [23]. The genotyping test is able to detect mutations associated with phenotypic resistance of HIV to ARVs [24]; it's efficacy has been demonstrated by several clinical and molecular studies [25]. We found that the male gender predominates among patients failing HAART. This was expected, since most patients initiated therapy in the mid 90's, when the male/female rate for HIV infection was 2.5 to 3 [26]. Due to increasing heterosexual transmission during the last years, it is conceivable that this rate, now 1.5, becomes close to 1.0 [27]. The median age of the group, 36 (± 4.5) years, is also similar to what has been reported for the country [28]. The median quantity of ARV regimens used, 3.0 (±0.8), demonstrates the importance of the genotyping test to help guide the choice of new regimens after treatment failure [14,18,19]. Many scientific reports have demonstrated the superiority of treatment decisions based on genotyping tests over other protocols [29].

The distribution of subtypes that we found was similar to findings from other Brazilian studies, characterized by a B subtype domain, followed by F and hybrid forms, involving B, F and D subtypes [30-32]. Although in expansion in our country, the absence of the C subtype in our sample is understandable given its low prevalence in the southeast region (the origin of all the samples that we analyzed) in comparison with the southern region of the country [33].

The number of samples presenting any resistance mutation (93.1%) was similar to what was found in other reports on patients failing HAART [34,35], although our rates were slightly elevated due to longer exposition to ARV drugs by this group of patients. This finding is of concern since resistance is a major impairment to therapeutic success [36]. NRTI drug resistance was much more frequent in comparison with resistance to other drug classes (p<0.001), occurring in 93.1% of the samples. The most prevalent: M184V (60.7%)

Table 1. Baseline characteristics of patients failing HAART attended by RENAGENO, and corresponding viral markers (N=117).

Patients	N	Viral markers	N
Gender		Viral load (median)	46,000 copies/mL
Male	74 (63.2%)	HIV-1 subtype	
Female	43 (36.8%)	B	86 (73.5%)
Age range (median ± SD)	35-48 (36±4,5)	F	7 (6%)
CD4 (median)	269 cells/mm^3	Hybrid	7 (6%)
No. of therapeutic regimens		Not determined	14 (12%)
Range (median ± SD)	1-7 (3±0.8)	Samples with resistance mutations	109 (93.1%)
Period treatment began N (%)		Samples with secondary mutations	117 (100%)
1988-1991	2 (1.7)	Presence of resistance-associated mutations	
1992-1995	6 (5.1)	RT gene	109 (93.1%)
1996-1999	44 (37.6)	PR gene	48 (41.0%)
2000-2004	22 (18.8)	Wild type	8 (6.48%)

Table 2. Antiretroviral drug efficacy affected by resistance mutations found in samples from patients failing HAART (N=117).

Resistance-associated mutations*	N (%)	Drug affected
NRTI		
M184V	71 (60.7)	3TC, ABC
T215Y	58 (49.6)	ZDV, d4T
M41L	43 (46.7)	ZDV, d4T
L210W	36 (30.8)	ZDV, d4T
D67N	30 (25.6)	ZDV, d4T
K70R	22 (18.8)	ZDV, d4T, TDF
I74V	10 (8.5)	ABC, ddI
K219Q	10 (8.5)	ZDV, d4T
Q151M	5 (4.3)	ABC
69 insertion	5 (4.3)	All NRTIs
NNRTI		
K103N	32 (27.3)	DLV, EFV, NVP
G190A	16 (13.6)	EFV, NVP
Y181C	13 (11.1)	DLV, EFV, NVP
L100I	10 (8.5)	EFV, NVP
Y188L	9 (7.7)	DLV, EFV, NVP
V108I	3 (2.6)	EFV, NVP
PI		
L90M	23 (19.6)	NFV, SQV
M46I	19 (16.2)	IDV, RTV
D30N	15 (12.8)	NFV
N88D	15 (12.8)	ATZ, RTV
I54V	12 (10.2)	Darunavir, RTV
I84V	10 (8.5)	SQV, RTV
L33F	3 (2.5)	Tipranavir, RTV
I50V	1 (0.85)	Darunavir, RTV, Fosamprenavir, ATZ

*: According to The International Aids Society 2006. NRTI: nucleosidic inhibitor of reverse transcriptase; NNRTI: non- nucleosidic inhibitor of reverse transcriptase; PI: protease inhibitor; 3TC: lamivudine; ABC: Abacavir; ZDV: Zidovudine; d4T: stavudine; TDF: Tenofovir; ddI: Didanosine; DLV: Delavirdine; EFV: Efavirenz; NVP: Nevirapine; NFV: Nelfinavir; SQV: Saquinavir; RTV: Ritonavir; ATZ: Atazanavir.

and T215Y (49.6%), responsible for resistance to 3TC/ABC and ZDV/d4T, respectively, indicates the common use of such drugs in therapeutic regimens [35]. K103N was the fifth most common mutation found (27.3%); it accounts for group-characteristic cross-resistance mutations to all available NNRTIs, indicating the growing use of such drugs as part of current HAART treatments.

PI resistance mutations (41.0%) were less frequent than RT inhibitor mutations, as expected, due to a natural genetic PR barrier to mutations [11], and because of more recent

Table 3. Number of resistance mutations detected in samples collected from patients failing HAART, grouped according to therapeutic scheme failure.

No. of therapeutic scheme failures[1]	No. of patients/scheme	No. of resistance mutations/sample[2] mean (SD)
1	16	3.18 (1.75)
2	24	4.39 (2.27)
3	34	4.40 (2.59)
4	20	3.94 (2.19)
5	17	4.0 (2.72)

[1]Only two patients were submitted to six and seven therapeutic schemes. [2]One sample per patient.

introduction of these drugs in therapy and use of alternative PIs in HAART regimens [34]. In contrast, secondary mutations, such as L63P (47%), L10I/F (34.2%), V77I (29%), A71V/T (26.5%) and M36I (21.3%) were common in the PR gene samples. The number and pattern of such mutations was similar to that found in other studies of patients failing HAART [37,38], though they may occur at similar rates in previously untreated patients [3]. These mutations configure polymorphisms that are not related to genetic resistance but are responsible for improving viral fitness [39].

The increasing number of resistance mutations from the first to the second drug failure indicates that HAART depends on the success of first regimens in order to avoid the appearance of HIV resistance variants [40]. The unexpected stabilization of resistance mutations, even after subsequent treatment failures, implies that HIV acquires a considerable number of key resistance mutations soon after treatment begins, and then stabilizes, probably avoiding further accumulation of resistance mutations that would impair subsequent viral replication [37]. This information is relevant to understanding HIV replication dynamics and sustained HAART effectiveness, although more studies that follow up patients would be necessary to support such a statement.

In conclusion, the high prevalence of HIV-1 resistance mutations and its complex genetic development during the course of ARV treatment, especially mutations against RT inhibitors, supports the necessity of sustained genotyping studies and carefully monitoring patients failing HAART.

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